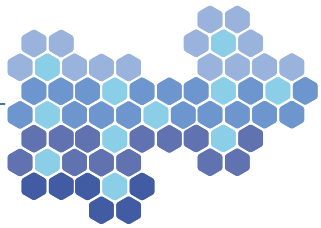


Challenges in Chromatography

Processing times can be reduced and yields increased by utilising new hydrophobic interaction chromatography tools

By Dr Nandu Deorkar at Avantor

Advances in protein and peptide engineering continue, enabling the creation of new biological products with improved therapeutic and diagnostic potential. Bringing these promising biologics to market effectively depends, in part, on solving significant downstream purification process challenges – such as separation of closely related impurities – that are the result of the increased complexity of producing these biologics.

Platform-based chromatographic approaches have been successful in separating and purifying monoclonal antibody products, but the newer generations of biologics feature different hurdles for purification process developers to overcome. Product- and process-related impurities, including host cell protein, aggregates, and charge and glycosylation variants, are persistent and often difficult to separate. As a result, process developers are increasingly exploring mixed-mode and multimode chromatographic media to achieve these separations.

Keywords

Biologics
Hydrophobic interaction chromatography
Mixed-mode media

Hydrophobic interaction chromatography (HIC), which is done by separating molecules based on their hydrophobicity, is one of these mixed-mode processes. Bioprocessors are working with HIC to address some of the process efficiency challenges facing the industry by fine-tuning the selectivity of HIC functional groups, thus potentially increasing yields and reducing the time to complete upstream separation and purification processes.

One avenue being explored is to use a select range of additives in the chromatographic media to improve the retention and selectivity of proteins as they move through the media, modulating their hydrophobic interaction. A series of studies were recently done to investigate the potential to improve HIC processes by modulating ligand- and protein-binding interaction through the use of a novel proprietary additive at different concentrations with a poly hi-propyl mixed-mode HIC media. The HIC media used in the studies is based on spherical polymethacrylate and silica beads, which are covalently bonded to polyethylenimine (PEI) and functionalised with propyl groups to offer mixed-mode functionalities.

Challenges of New Biopharmaceutical Production

Biopharmaceutical producers are working to reduce variability in manufacturing processes to minimise drug product heterogeneity. In antibody purification, separating undesired glycosylated molecules, heterogeneous molecules (product variants), and aggregates presents major challenges, as they are likely to have limited differential binding to traditional ion exchangers and can coelute. The traditional solution to this challenge is to implement multiple separations based on simple ion-exchange chromatographic media.

More advanced methods of achieving effective selectivity are based on new ligand chemistries engineered to achieve precise selective interactions with the targeted protein. Targeted affinity chromatographic media are based on ligands tailored to interact with specific proteins, offering high selectivity for a target drug molecule. HIC is a useful separation technique for purifying proteins while maintaining biological activity due to the use of conditions and matrices that operate under less denaturing conditions. While this tactic offers the advantages of increased selectivity, it can be a time-consuming approach if implemented for every new type of molecule.

Tuning Selectivity Through Organic Additives

The efficiency of HIC can be tuned through manipulating certain

Elution chromatogram

Conditions:
Column: 100 X 4.6mm ID
Linear velocity: 361cm/h

Binding buffer: 25mM NaH_2PO_4, pH = 7.0 $(NH_4)_2SO_4$

Elution buffer: 25mM NaH_2PO_4, pH = 7.0
Linear gradient time: 40 min

Samples:
Cytochrome (~1mg/ml)
Myoglobin (~2mg/ml)
Lysozyme (~2mg/ml)
α-Amylase (~3mg/ml)

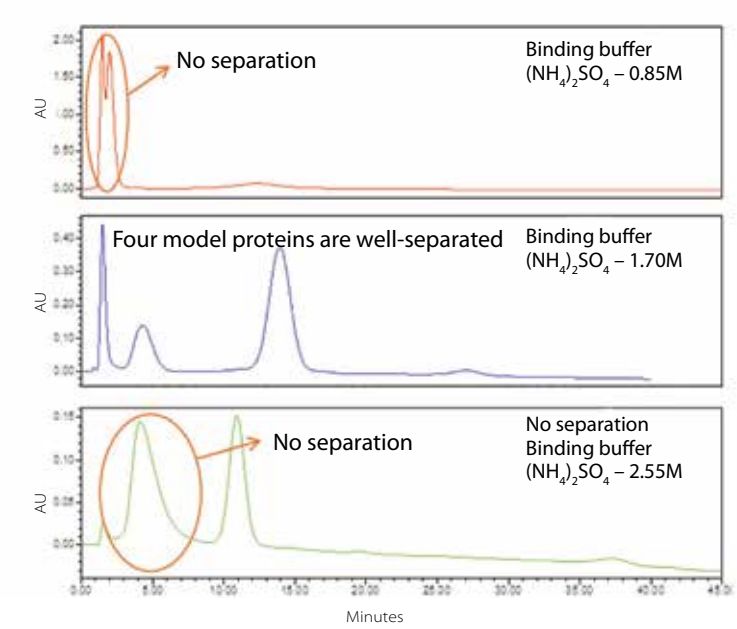


Figure 1: Optimal salt concentration of protein separation by poly hi-propyl. Three concentrations of ammonium sulfate were tested with four target proteins. 1.70M of $(NH_4)_2SO_4$ provides better selectivity on poly hi-propyl

Elution chromatogram

Conditions:
Column: 100 X 4.6mm ID
Linear velocity: 361cm/h

Binding buffer:
25mM NaH_2PO_4, pH = 7.0
1.7M $(NH_4)_2SO_4$

Elution buffer:
25nM NaH_2PO_4, pH = 7.0
Additive: AD1
Linear gradient time:
40 min

Samples:
Cytochrome (~1mg/ml)
Myoglobin (~2mg/ml)
Lysozyme (~2mg/ml)
α-Amylase (~3mg/ml)

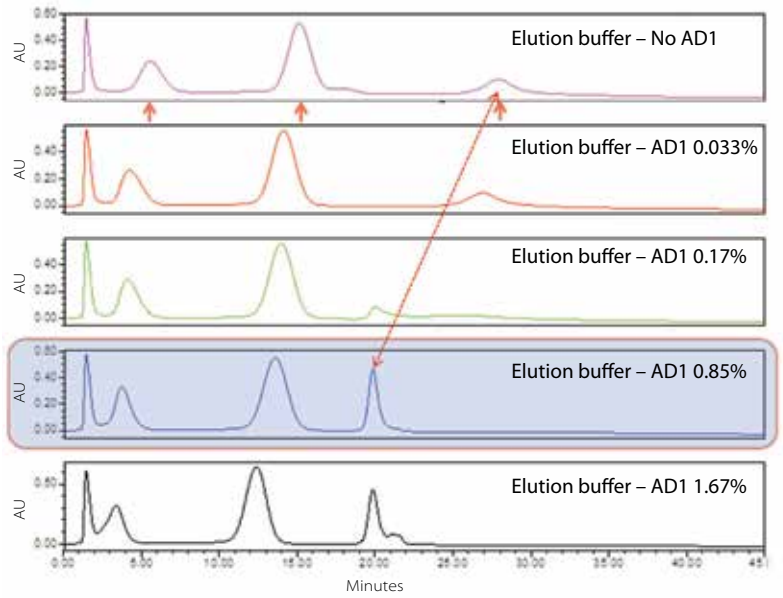


Figure 2: Effect of Additive 1 (AD1) on protein separation. The best result was with 0.85M concentration of the additive

environmental conditions in the media, including buffer or salt type, concentration of additives, pH levels, and other factors. Since HIC tends to aggregate proteins due to enhanced intermolecular hydrophobic interaction, HIC's advantage over traditional ion-exchange chromatography methods can be somewhat diminished in terms of overall yield.

To address this issue, bioprocessors have turned to using organic additives to improve HIC efficiency. Commonly used additives, such as ethanol and isopropanol, have been shown to modulate the interactions between proteins and HIC functional groups. This can tune the selectivity and retention of the targeted protein.

The addition of isopropanol (up to 13%) can effectively reduce the retention and improve the separation selectivity of five monoclonal antibodies (mAbs). However, since additives like isopropanol and ethanol are organic solvents, they can damage the unfolding of the targeted protein, thereby impacting the chromatography yield. Also, organic solvents require special handling for safety purposes, which can add to overall process costs for storage, training, documentation of procedures, and other issues.

Addressing these limitations are, in part, what led to the investigation of how alternative additives like salts or buffers could improve HIC efficiency and potentially replace organic

Figure 3: The AD1 additive dramatically improves HIC process efficiency for the target molecule by reducing elution volume on poly hi-propyl

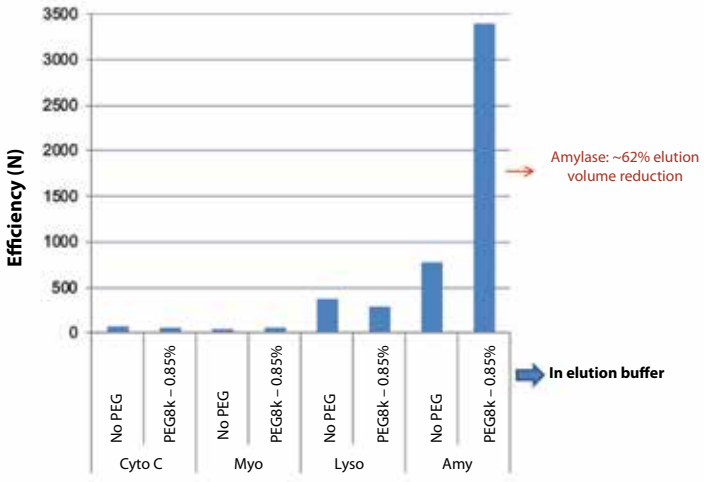
Conditions:
Column: 4.6 x 100mm

Linear velocity: 361cm/h

Binding buffer:
25mM Na_H2PO_4, 1.7M $(NH_4)_2SO_4$
pH = 7.0

Elution buffer:
25mM NaH_2PO_4, pH = 7.0

Linear gradient time: 40 min



solvents as HIC additives. In this case, three facets were studied:

- Potential for HIC process improvement that might result from modulating ligand and protein interaction induced by various buffer additives at different concentrations on mixed-mode HIC media
- Effect of functional groups on selectivity of model proteins
- Application of additives for better mAb separation

The primary hydrophobic interaction is due to the propyl groups covalently bonded to PEI and secondary interactions provided by the amino groups on PEI, offering enhanced selectivity and high efficiency. Mixed-mode chromatography media are based on ligands that offer two or more interaction possibilities with the targeted drug molecule.

Specific Investigations of Alternative Additives

The first additives to be researched were salts – specifically ammonium sulfate. The addition of salts is generally known to affect protein interaction in HIC processes, but the fine-tuned interaction is not well-studied.

The effect of ammonium sulfate concentration on protein selectivity in HIC media was studied, with three concentrations tested: 0.85M, 2.55M, and 1.70M. Four target proteins were studied: Cytochrome c (~1mg/ml), myoglobin (~2mg/ml), lysozyme (~2mg/ml), and α-amylase (~3mg/ml) (see Figure 1, page 9).

Although it was determined that the 1.70M of ammonium sulfate provides better selectivity on the poly hi-propyl HIC media, the selectivity improvement was not significant.

Next, a series of studies were conducted on a novel additive, referred to as Additive 1 (AD1). AD1 is a proprietary molecule that has water activity change properties, removing the water molecule either from the protein surface or from the chromatographic reagent surface, to help drive the hydrophobic interaction.

The same four target molecules were studied, and a range of AD1 concentrations were used to compare the effect of AD1 on protein separation – four different concentrations, plus one pass that contained no additive. The most significant finding was that the elution peak started earlier in the 40-minute run with the AD1 additive compared to later in the run with no additive. The best result was with the 0.85M concentration of the additive (see Figure 2, page 9).

The most dramatic impact of using the AD1 additive occurred in the process efficiency for α-amylase. It was shown to reduce amylase retention factor without affecting protein separation factor with the HIC media. Traditionally, without the use of additives, amylase hits peak elution later in the chromatographic process, usually at the 40- to 50-minute point.

With the additive, the retention time is reduced to almost 30 minutes, offering the potential for much faster separation. It was shown that, for amylase, this faster separation does not affect the volume of separation. This concluded that the AD1 additive dramatically improves HIC process efficiency for the target molecule (see Figure 3).

Given that the additive only improved the HIC efficiency for one protein, whereas minimal to no impact occurred on the efficiency of the other three proteins studied, it is apparent that this type of additive could demonstrably be highly selective to specific proteins. This theory is worth further study.

A second series of studies was conducted to assess the impact of different molecular weights of the AD1 additive on protein separation with the poly hi-propyl HIC media. A range of molecular weights was studied, and the AD1 at 0.85% was found to show the most profound

Conditions:
Column: 100 X 4.6mm ID

Linear velocity: 361cm/h

Binding buffer:
Elution buffer + 1.0M $(NH_4)_2SO_4$

Elution buffer:
25mM NaH_2PO_4, pH = 7.0

Linear gradient time: 40 min

Samples:
mAb loaded: 3.6mg
(0.4mg/ml) in binding buffer

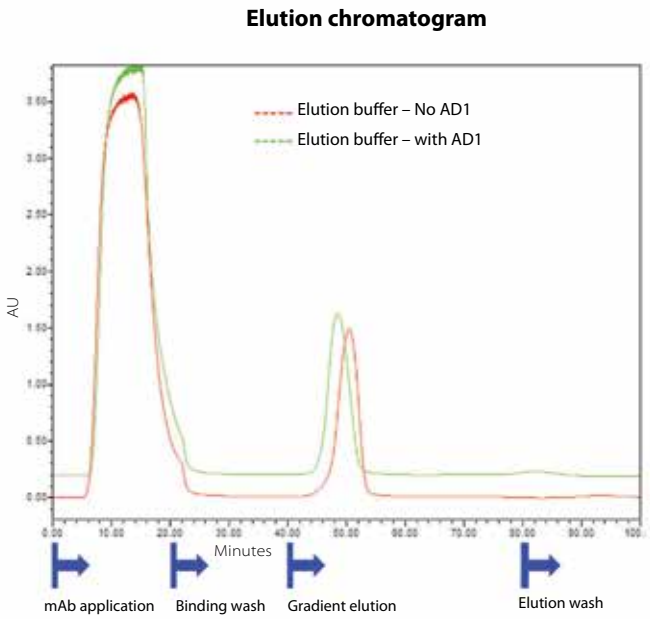


Figure 4: Purification of a mAb from clarified cell culture feed stock by HIC using poly hi-propyl media with additive

effect on protein separation, in terms of retention ability and efficiency. At this point, the conclusion can be drawn that there is no significant difference, and it would be possible to use both lower and higher concentrations without significantly impacting HIC efficiency – at least with the target protein amylase.

Different concentrations of the AD1 additive were also studied, with conclusions similar to the molecular weight results. Thus, a correlation was established between the additive concentration, molecular weight, and the target protein in terms of the additive's ability to improve HIC efficiency.

A final study was conducted to see the potential impact of using the AD1 additive with the poly hi-propyl media to process monoclonal antibodies. In this study, the mAb feedstock was a clarified cell culture broth. It was a one-step mAb purification, with high purity and recovery. HIC using the poly hi-propyl was done with, and without, the AD1 additive (see Figure 4).

Significantly better results were found using the additive. Without it, mAb recovery was 95.4% and purity was 97%; with the AD1 additive (using size-exclusion chromatography), recovery jumped to 101.6%, and purity increased to 98% (see Table 1).

	% recovery	mAB purity*
Run – with AD1	101.60%	98%
Run – No AD	95.40%	97%

*By size-exclusion chromatography

Table 1: Significantly better results were found using the AD1 additive in HIC mAb purification

New Approaches

Within the chemical industry, significant (and valuable) emphasis has been placed on improving process chromatography resins in terms of their sophistications and selectivity. With this study, clear evidence suggests that also exploring the potential for using additives, beyond the traditional isopropyl/ethanol categories, can offer substantial avenues for progress.

These studies of the value of the novel additive AD1, when used with HIC media, demonstrate:

- Functional groups attached to HIC play critical roles on protein separation and efficiency
- The selected additives at specific concentrations improve the HIC process by improving separation efficiency and decreasing retention time, thereby improving process throughput
- Both molecular weights and their concentrations of additives play critical roles on protein separation in HIC

Rather than focussing on the process chromatography resin, alternatives, such as additives and buffers, can improve HIC efficiency and provide biopharma companies with new tools to potentially reduce downstream processing time and increase yields.

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